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Purification and Properties of Aminoendopeptidase from Rat Epidermis

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An aminoendopeptidase isolated from 2-day-old rat epidermis was purified to apparent homogeneity by the procedures of ammonium sulfate fractionation, DE-52 column chromatography, Sephadex G-200 gel filtration, and CM-52 and DEAE-Sepharose 6B column chromatography. Enzymatic activity was exhibited only in the presence of sulfhydryl compounds and further enhanced by addition of 5 mM EDTA. It was inhibited by *p*-chloromercuribenzoate, other sulfhydryl blocking reagents, and *o*-phenanthroline. The monomer form of the enzyme is $M_r = 52{,}000 \pm 2{,}300$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, but a native form was considered to be $M_r = 400{,}000 \pm 26{,}000$ having an isoelectric point of pH 5.25. Among synthetic substrates the enzyme hydrolyzed amino acid 2-naphthylamide derivatives and L-leucine amine (L-LeuNH$_2$) most effectively. N-α-benzoyl-DL-arginine-2-naphthylamide (BANA) was the only endopeptidase substrate for the enzyme and a competitive inhibitor for its aminopeptidase activity. Protein substrates have not yet been found. The pH optimum is 7.5 and in a range of pH 6.5–7.5 it is stable at 37°C for 30 min but loses about 50% of its activity at 50°C.

Epidermal cells of mammalian skin turn over in an extremely harmonious fashion and both synthetic and hydrolytic actions are involved in cell cornification and desquamation. Many hydrolytic enzymes are considered to participate in the regulation of epidermal cell function (e.g., [1,2]). Hashimoto et al [3] reported that autodegradation of Triton X-100-soluble epidermal proteins was primarily caused by endogenous cysteine proteinase(s). However, sulfhydryl-activated proteases investigated so far have come from whole-skin preparations [4–9] and their relation to epidermal cells remains unknown, although a cysteine proteinase inhibitor was purified from the epidermis of rat [10,11] and human [12,13].

Previously we extracted a protease capable of hydrolyzing both N-α-benzoyl-DL-arginine-2-naphthylamide (BANA) and L-leucine-2-naphthylamide (Leu-βNA) from cornified cells of 2-day-old rats [14]. This aminoendopeptidase was not detectable without sulfhydryl compounds and the activity was inhibited by *p*-chloromercuribenzoate. Since the hydrolase showed a number of properties that distinguish it from sulfhydryl-dependent aminoendopeptidases characterized in whole skin of rat [7,8] and human [6], rat liver and kidney [15,16], rabbit lung [17], and bovine spleen and human placenta [18], we conducted further characterization of the epidermal hydrolase after purification. In this report the purification procedure and the properties, including substrate specificity, are described.

MATERIALS AND METHODS

Materials

Two-day-old Sprague-Dawley strain rats were used. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman. DEAE-Sepharose 6B and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. Synthetic substrates were from Sigma Chemical Co., and hemoglobin and casein were from Miles Co. All other chemicals were obtained from commercial sources and were of the purest grade available.

Enzyme Assay

Epidermal aminoendopeptidase activity was assayed by a minor modification of the method of Järvinen and Hopsu-Havu [7]. The enzyme was activated by incubation of epidermal samples in 1 ml of 0.1 M Tris-HCl buffer, pH 7.5, 5 mM dithiothreitol, and 5 mM EDTA at 37°C. After 5 min 20 μl of 0.1 M Leu-βNA or BANA dissolved in dimethylsulfoxide was added and reaction continued at 37°C for 10–60 min. The reaction was stopped by addition of 1 ml of 25% acetic acid in ethanol (v/v) and 1 ml of 0.2% *p*-dimethylaminocinnamaldehyde in ethanol (w/v). The color was developed for 15 min at 37°C, and was measured at 545 nm. One unit of enzyme activity was defined as the amount which hydrolyzed 1 μmol substrate per min. Substrate specificity and enzyme kinetics of various amino acid 2-naphthylamide derivatives were studied with the same assay system.

Purification of Epidermal Aminoendopeptidase

The skin was removed from rats and the epidermis peeled off from the dermis after immersion in 0.24 M NH_4Cl, pH 9.5, for 10 min. Cornified cells (about 10 g) scraped from the epidermis of 200 rats were pooled and washed 5 times with 100 ml of 10 mM Tris-HCl buffer, pH 7.5, 0.14 M NaCl. They were homogenized in 100 ml of 20 mM sodium phosphate buffer, pH 6.0, 0.14 M NaCl and stirred for 2 h. The supernatant was obtained by centrifugation at 25,000 *g* for 20 min. The extraction procedure was repeated, and the two supernatants were pooled. Solid ammonium sulfate was added slowly to the crude extract of cornified cells with stirring until 40% saturation was achieved. The suspension was incubated on ice for 30 min after all ammonium sulfate was in solution and was centrifuged at 25,000 *g* for 30 min. The supernatant was removed, adjusted to 50% saturation with ammonium sulfate, and centrifuged as before. The pellet was dissolved in and dialyzed against 20 mM sodium phosphate buffer, pH 6.0. This fraction is referred to as the 40–50% ammonium sulfate fraction. A DE-52 column (1.5 × 15 cm) was prepared and equilibrated with 20 mM sodium phosphate buffer, pH 6.0. The 40–50% ammonium sulfate fraction was adsorbed onto the column at a flow rate of 1 ml/min. The column was washed with 150 ml of the equilibration buffer and elution was done with a 400-ml linear gradient from 0–0.4 M NaCl in the equilibration buffer at a flow rate of 1 ml/min.

The fractions with hydrolase activity for both Leu-βNA and BANA eluted in the concentration of approximately 0.1 M NaCl were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 6.0. It was applied to a CM-52 column (1.5 × 15 cm), equilibrated with 20 mM sodium phosphate buffer, pH 6.0, and eluted with the same buffer at a flow rate of 1 ml/min. Almost all enzyme activity (mU/ml) was passed through. The active fraction was concentrated to 3 ml with PM-10 (Amicon) and applied on a Sephadex G-200 column (1.5 × 83 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, 0.1 M NaCl. The fractions with hydrolase activity were collected, dialyzed against 20 mM Tris-HCl buffer, pH 7.5, and adsorbed onto a DEAE-Sepharose 6B column (1.5 × 10 cm) equilibrated with the same buffer, at a flow rate of 1 ml/min. The column was washed with 150 ml of the equilibration

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Abbreviations:
BANA: N-α-benzoyl-DL-arginine-2-naphthylamide
L-LeuNH$_2$: L-leucine amine
SDS: sodium dodecyl sulfate
TCA: trichloroacetic acid

TABLE I. *Summary of the purification of aminoendopeptidase from rat epidermis*

Purification step	Total volume (ml)	Total protein (mg)	Leu-βNA hydrolytic activity			BANA hydrolytic activity		
			Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)
Crude extract	193	326	14,100	43.3	100	344	1.06	100
40–50% Ammonium sulfate fraction	40	15.2	7,660	504	54.3	183	12.0	53.2
DE-52 chromatography	45	3.68	5,890	1,600	41.8	142	38.6	41.3
CM-52 chromatography	10	3.07	5,450	1,780	38.7	121	39.4	35.2
Sephadex G-200 chromatography	18	1.36	4,110	3,020	29.1	97.8	71.9	28.4
DEAE-Sepharose 6B chromatography	35	1.10	4,050	3,680	28.7	92.5	84.1	26.9

buffer. Elution of the column was performed with a 400-ml linear gradient from 0–0.4 M NaCl in the equilibration buffer at a flow rate of 1 ml/min. The aminoendopeptidase eluted at a NaCl concentration of 0.15 M was used as the purified enzyme preparation. In some experiments dithiothreitol was added to the solutions used for extraction and purification at the final concentration of 0.2 mM.

Polyacrylamide Gel Electrophoresis

Disc polyacrylamide gel electrophoresis was performed in 7.5% gel at pH 9.4 according to the method of Davis [19]. The gel was stained with Coomassie Blue R-250 and for activity with Leu-βNA and BANA as substrates by the method of Barrett and Kirschke [20]. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out in 7.5% gel by the method of Weber and Osborn [21].

Isoelectric Point

The ampholines pH 3.5–10 were used at a final concentration of 1% and isoelectric focusing was run in an LKB Ampholine Column 8100 (110 ml). The sucrose gradient was established by mixing proper proportions of dense- and light-solution fractions exactly as described in the LKB manual. The purified aminoendopeptidase was dialyzed against 0.5% carrier ampholytes and introduced approximately in the middle of the column. The anode at the bottom contained phosphoric acid and the cathode at the top contained ethanolamine as electrode solutions. The focusing was accomplished at 4°C with 300 V for the first h followed with 900 V for 48 h.

Protein Determination

Protein was determined by the method of Lowry et al [22], with bovine serum albumin as the standard.

Hydrolysis of Synthetic Substrates

The purified aminoendopeptidase was activated in 0.2 ml of 0.2 M sodium phosphate buffer (pH 7.5), 10 mM dithiothreitol, and 10 mM EDTA for 5 min at 37°C. The enzyme solution was then incubated with 0.2 ml of various synthetic substrates in H_2O for 60 min at 37°C and the reaction was stopped by addition of 1.6 ml of 0.2 M sodium citrate buffer, pH 5.0. The primary amino groups released from 1 mM L-LeuNH$_2$, L-Leu-L-Leu, L-His-L-Leu, L-Leu-Gly-Gly, Nα-benzoyl-L-ArgNH$_2$, hippuryl-L-Phe, and hippuryl-L-His-L-Leu were measured by the ninhydrin method [23]. Activities with 5 mM Nα-benzoyl-DL-Arg-*p*-nitroanilide and L-Leu-*p*-nitroanilide were determined at 410 nm [24]. Hydrolysis of 0.5 mM Nα-benzoyl-L-Arg ethyl ester was measured at 254 nm [25].

Digestion of Proteins

The purified aminoendopeptidase was activated in 0.25 ml of 0.2 M sodium phosphate buffer, pH 7.5, containing 20 mM dithiothreitol and 20 mM EDTA for 5 min at 37°C. Proteins tested were dissolved in 0.1 M sodium phosphate buffer, pH 7.5, at 2% (w/v) concentration and 0.5 ml of each protein solution was incubated with the enzyme for 24 h at 37°C. The reaction was stopped with 1.5 ml of 0.1 M trichloroacetic acid (TCA) solution at pH 4.0, prepared by dissolving 16.3 g of TCA, 16.4 g of anhydrous sodium acetate, and 18 ml of acetic acid in distilled water to make a final volume of 1 liter. After filtration of the incubation mixture through Whatman filter paper #1, 1 ml of filtrate was mixed with 5 ml of 0.55 M Na_2CO_3 and 1 ml of phenol reagent (2 × dilution) at 37°C for 30 min. Short-chain peptides released were measured using L-tyrosine as the standard at 750 nm.

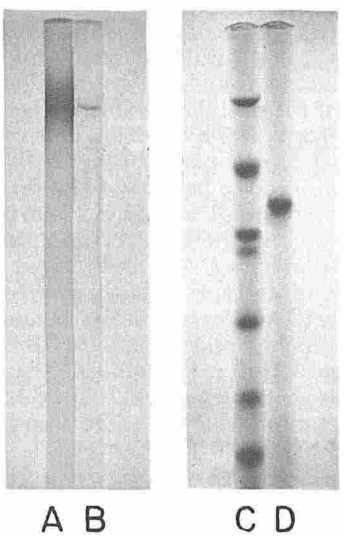

FIG 1. Disc (gels *A*, *B*) and SDS polyacrylamide gel (gels *C*, *D*) electrophoresis. Gels *A*, *B*, and *D* are aminoendopeptidase purified from rat epidermis. Gel *C* contains standard proteins (described in detail in Fig 2*B*). Gel *A* was stained for activity using Leu-βNA as the substrate, and gels *B*, *C*, and *D* were stained with Coomassie Brilliant Blue R-250.

RESULTS

Purification of Aminoendopeptidase from Cornified Cells

The yield of purification achieved in a typical preparation is summarized in Table I. A 50% reduction in total enzyme activity occurred by the ammonium sulfate fractionation although specific activity rose about 12-fold. Recovery of the hydrolase activity through the last purification step with DEAE-Sepharose 6B chromatography was approximately 30% and an 85-fold purification was accomplished. At all of the steps hydrolytic activities for Leu-βNA and BANA always appeared in the same fraction and the hydrolysis rate of Leu-βNA was about 40 times greater than that of BANA. By polyacrylamide gel electrophoresis at pH 9.4 on 7.5% gel, the purified enzyme gave a single protein band which demonstrated activity after the enzyme stain (Fig 1).

Physicochemical Properties of Epidermal Aminoendopeptidase

The $M_r = 400{,}000 \pm 26{,}000$ was estimated from gel filtration on a Sephadex G-200 column with or without dithiothreitol in the elution buffer (Fig 2*A*). However, electrophoresis of the purified enzyme on 7.5% gel in the presence of 0.1% SDS revealed one band and a $M_r = 52{,}000 \pm 2{,}300$ was calculated (Figs 1, 2*B*), and it was considered that the native form consists of an octamer of $M_r = 52{,}000$. By isoelectric focusing analysis, the isoelectric point of the hydrolase was found to be pH 5.25. The enzyme has a pH optimum of 7.5 and is stable at neutral pH at 37°C for 30 min, but is inactivated at both acidic and

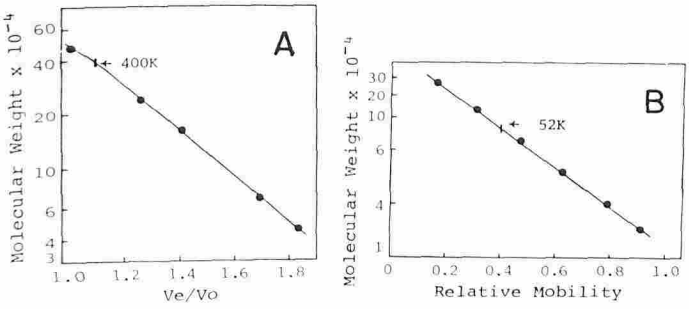

FIG 2. M_r calibration of epidermal aminoendopeptidase by Sephadex G-200 column chromatography (*A*) and SDS polyacrylamide gel electrophoresis (*B*). *A*, Semilog plot of M_r ($\times 10^{-4}$) vs Ve/Vo for aminopeptidase (*arrow*) and standard proteins (horse ferritin, M_r = 440,000; bovine catalase, M_r = 240,000; rabbit aldolase, M_r = 160,000; bovine serum albumin, M_r = 67,000; albumin, M_r = 45,000). *B*, Semilog plot of M_r ($\times 10^{-4}$) vs mobility relative to tracking dye in 7.5% SDS polyacrylamide gels for aminoendopeptidase (*arrow*) and several standard proteins (rabbit phosphorylase b, M_r = 94,000; bovine serum albumin, M_r = 67,000; ovalbumin, M_r = 45,000; bovine carbonic anhydrase, M_r = 30,000; soybean trypsin inhibitor, M_r = 20,100; α-lactalbumin, M_r = 14,000).

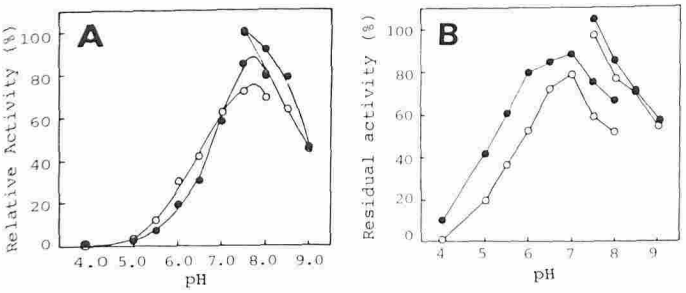

FIG 3. pH optimum and pH sensitivity of aminoendopeptidase. Buffers (0.1 M) used were: sodium acetate buffer, pH 4.0–5.5; sodium phosphate buffer, pH 6.0–8.0; Tris-HCl buffer, pH 7.5–9.0. They contained 5 mM dithiothreitol and 5 mM EDTA. *A*, The pH optima were found to be 7.5 for both Leu-βNA (○—○) and BANA (●—●). The activity was assayed by the standard method for both Leu-βNA (○—○) and BANA (●—●). *B*, Effects of the pHs were tested after treating the enzyme in these buffers at 37°C for 30 min. More than 75% of the original activity remained at a range of pH 6.5–8.0, but the enzyme was not stable below pH 6.0 or above pH 8.0.

alkaline pHs (Fig 3). The activity at neutral pH is maintained in Tris-HCl buffer more effectively than in phosphate buffer. Stability of the enzyme in pH 7.5 at 50°C is shown in Fig 4. The enzyme lost almost 80% of its native activity after 60 min. Rates of inactivation with time, determined by the use of Leu-βNA and BANA, were identical.

Effects of Various Compounds and Metal Ions on Epidermal Aminoendopeptidase

The activities for both Leu-βNA and BANA were not detected in the reaction mixture without dithiothreitol. Both substrates were hydrolyzed by addition of dithiothreitol at a 5 mM concentration, but the maximum activities were obtained in the reaction mixture containing both 5 mM EDTA and 5 mM dithiothreitol. By taking the maximum activities as 100, Table II compares the activities in a mixture prepared with different concentrations of dithiothreitol and EDTA. In the experiments, 5 mM glutathione was used to replace dithiothreitol; percent hydrolytic activities were 11% (without EDTA) and 71% (with 5 mM EDTA) for Leu-βNA, and 5% (without EDTA) and 54% (with 5 mM EDTA) for BANA.

p-Chloromercuribenzoate and iodoacetic acid are potent inhibitors but iodoacetamide and N-ethylmaleimide inhibited the hydrolase activities to a lesser extent (Table III). While leupeptin, pepstatin, and antipain showed almost no effects on the hydrolase, puromycin moderately inhibited its activity for BANA but not Leu-βNA. Phenylmethylsulfonyl fluoride was an inhibitor, but diisopropyl fluorophosphate was not. Other compounds known to inhibit serine proteases, including α_1-antitrypsin, soybean trypsin inhibitor, Tos-Lys-CH_2Cl, and Tos-Phe-CH_2Cl, showed no effects. KCN activated the activity only for BANA. While EDTA stimulated the activity, *o*-phenanthroline was a strong inhibitor.

Powerful inhibition by Hg^{2+} was seen even though the activity was measured in the presence of EDTA. Other metal ions, Cu^{2+}, Zn^{2+}, and Cd^{2+}, moderately inhibited the activities for both Leu-βNA and BANA. Fe^{2+} and Mn^{2+} had almost no effect on the activity for Leu-βNA but inhibited BANA hydrolysis. On the other hand, Ca^{2+}, but not Mg^{2+}, stimulated the activity for Leu-βNA but not for BANA. In separate experiments we examined Ca^{2+} effects under the presence and absence of EDTA (Fig 5). Ca^{2+} activation occurred only in the presence of EDTA, and increased Leu-βNA activity, up to 170% with 10 mM Ca^{2+}, was seen. BANA hydrolysis also was slightly activated with 1–2 mM Ca^{2+} but it was decreased at higher concentrations. However in the absence of EDTA, Ca^{2+} inhibited aminoendopeptidase activity.

Substrate Specificity

K_m and V_{max} values obtained for 2-naphthylamide derivatives are compared in Table IV. The most sensitive substrates for aminopeptidase activity occurred in the following order: serine-, lysine-, glycine- and leucine-βNA, while BANA was less effective as a substrate than amino acid 2-naphthylamides.

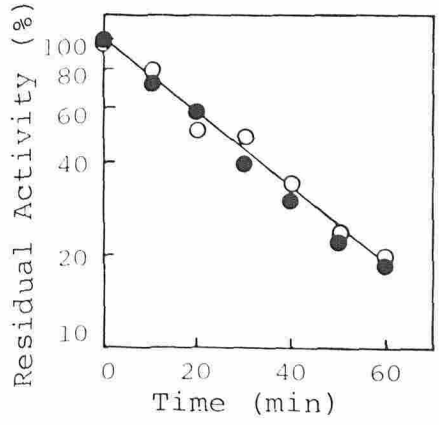

FIG 4. Stability of epidermal aminoendopeptidase at 50°C. The enzyme was preincubated at 50°C for the indicated times in 0.1 M Tris-HCl buffer, pH 7.5. The residual enzyme activity was assayed by the standard method using Leu-βNA (○—○) and BANA (●—●).

TABLE II. *Effect of dithiothreitol and EDTA on aminoendopeptidase from rat epidermis*

Concentration (mM)		Relative activity (%)	
Dithiothreitol	EDTA	Leu-βNA	BANA
0	0	0	0
5	0	76	38
5	1	98	71
5	5	100	100
5	10	102	98
0	5	8	12
1	5	67	88
10	5	101	100

The hydrolase was preincubated at 37°C for 20 min in 0.1 M Tris-HCl buffer, pH 7.5, containing different final concentrations of dithiothreitol and/or EDTA. Leu-βNA and BANA were used as substrates.

TABLE III. *Effect of various compounds and metal ions on aminoendopeptidase from rat epidermis*

Compound	Conc. (mM)	Relative activity (%)	
		Leu-βNA	BANA
None		100	100
p-Chloromercuribenzoate	1	0	0
Iodoacetamide	1	13	29
Iodacetic acid	1	4	6
N-Ethylmaleimide	1	37	29
Diisopropylfluorophosphate	1	97	100
Phenylmethylsulfonyl fluoride	1	50	57
Puromycin	1	95	50
Leupeptin	0.002	99	105
Pepstatin	0.01	100	106
Antipain	0.001	99	113
α_1-Antitrypsin	0.1 mg/ml	103	139
Soybean trypsin inhibitor	0.1 mg/ml	105	115
Tos-Lys-CH_2Cl[a]	0.01	103	113
Tos-Phe-CH_2Cl[b]	0.01	104	117
KCN	1	111	150
o-Phenanthroline	1	6	0
Mg^{2+}	1	90	88
Ca^{2+}	1	141	116
Mn^{2+}	1	88	23
Fe^{2+}	1	97	16
Co^{2+}	1	100	93
Ni^{2+}	1	101	100
Cu^{2+}	1	53	41
Zn^{2+}	1	58	71
Cd^{2+}	1	56	59
Hg^{2+}	1	0	0

The purified hydrolase was preincubated at 37°C for 20 min in 0.1 M Tris-HCl buffer containing 5 mM dithiothreitol and 5 mM EDTA, pH 7.5, with one of the compounds or metal ions at the final concentrations indicated. The hydrolytic activities for Leu-βNA and BANA were then measured as described in the text. The activities were expressed as the percent of those without the modifier in preincubation. All nonliquid chemicals were dissolved in distilled water except stock solutions of PMSF (20 mM) and pepstatin (5 mM), which were prepared in methanol. Appropriate controls for vehicles were included.

[a] Nα-*p*-tosyl-L-lysine chloromethyl ketone.

[b] L-1-tosylamide-2-phenyl chloromethyl ketone.

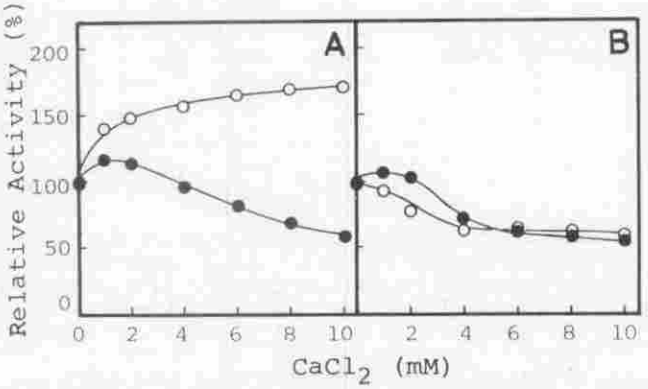

FIG 5. Effect of Ca^{2+} on epidermal aminoendopeptidase. The enzyme was preincubated for 15 min at 37°C in 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM dithiothreitol and various concentrations of $CaCl_2$ in the presence (*A*) and absence (*B*) of 5 mM EDTA.

Hydrolytic activity for dipeptides, tripeptide, and aminopeptidase substrates other than amino acid 2-naphthylamide, as compared to that for Leu-βNA, are presented in Table V. L-LeuNH_2 and Leu-βNA were found to be most sensitive. Synthetic substrates for endopeptidase, other than BANA, and including Nα-benzoyl-L-argininamide, hippuryl-L-phenylalanine, hippuryl-L-histidyl-L-leucine, Nα-benzoyl-DL-arginine-*p*-nitroanilide, N-benzoyl-L-tyrosine-*p*-nitroanilide, and Nα-benzoyl-L-arginine ethyl ester were not substrates for the aminoendopeptidase. In order to demonstrate that both aminopeptidase and endopeptidase are catalyzed by the same enzyme molecule, competitive inhibition experiments were carried out as reported by Singh and Kalnitsky [26]. Fig 6 shows that BANA inhibited competitively the aminoendopeptidase activity for L-Leu-*p*-nitroanilide.

As a protein substrate we examined γ-globulin (bovine), hemoglobin (bovine), α-casein, azocasein, calf histone, histone (calf, arginine-rich), histone (calf, lysine-rich), and bovine

TABLE IV. *Kinetic constants of epidermal aminoendopeptidase toward 2-naphthylamide derivatives*

2-Naphthylamide derivative	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/mM)
Leu-βNA	0.357	4.14	11.60
Ala-βNA[a]	0.526	3.31	6.29
Arg-βNA	0.319	3.95	12.38
Cystine-di-βNA	0.250	0.50	2.00
Gly-βNA	0.469	4.72	10.06
Ile-βNA	0.351	0.90	2.56
Lys-βNA	0.392	4.76	12.14
Phe-βNA	0.546	1.58	2.89
Pro-βNA	—	0.21[b]	—
Ser-βNA	0.500	6.00	12.00
Trp-βNA	—	0.33[b]	—
Tyr-βNA	—	0.33[b]	—
Val-βNA	0.469	0.88	1.88
BANA	0.339	0.07	0.21

The purified enzyme (0.2 μg) was incubated with different concentrations of 2-naphthylamide derivatives in the presence of 5 mM dithiothreitol and 5 mM EDTA, and activity measured as described in the text. Values of K_m and V_{max} were obtained from Lineweaver-Burk plots.

[a] βNA, 2-naphthylamide.

[b] The values present activities measured with the substrates at 0.5 mM concentration.

TABLE V. *Substrate specificity of aminoendopeptidase from rat epidermis*

Substrate	Relative activity (%)
Leu-βNA	100
L-Leu-*p*-nitroanilide	68
L-LeuNH_2	102
L-Leu-L-Leu	15
L-His-L-Leu	2
L-Leu-Gly-Gly	46

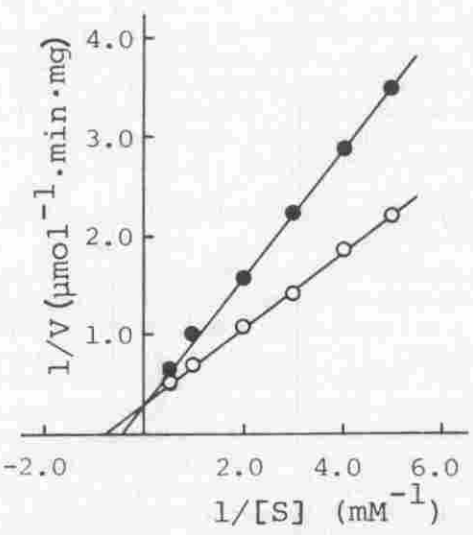

FIG 6. Competitive inhibition of aminopeptidase activity of epidermal aminoendopeptidase by BANA. The purified enzyme was incubated with different concentrations of L-Leu-*p*-nitroanilide in 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM dithiothreitol and 5 mM EDTA in the absence (○—○) and presence (●—●) of 0.5 mM BANA.

serum albumin. None of those was found to be a suitable substrate.

DISCUSSION

A hydrolase having both aminopeptidase and endopeptidase activities was isolated from the cytosol fraction of cornified cells of 2-day-old rats. The apparent purity of the enzyme was demonstrated by disc electrophoresis and SDS gel electrophoresis. However physicochemical analysis of the binding site(s) with Leu-βNA and BANA was not included.

Sulfhydryl compounds were absolute requirements for the hydrolytic activities which were effectively inhibited by *p*-chloromercuribenzoate and iodoacetic acid. Substrates with a free α-amino group were hydrolyzed much faster than BANA, a substrate with a blocked α-amino group. BANA hydrolases with similar properties were purified from rat whole skin by Järvinen and Hopsu-Havu [7] and further characterized by Järvinen [8], but they differ from the epidermal aminoendopeptidase by M_r, pH optima, and substrate specificity. BANA hydrolase from whole skin has a $M_r = 27{,}000$ and pH optimum of 5.8 for BANA and 7.0 for Leu-βNA. Activity of the whole-skin enzyme increased after 50°C incubation at pH 4.0, while the epidermal aminoendopeptidase activity was rapidly lost. A sulfhydryl-dependent neutral hydrolase in rat skin studied by Mäkinen and Raekallio [27] appeared to have a small M_r, and soluble 2-naphthylamidases of human and guinea pig epidermis characterized by Gray et al [28] showed inhibition profiles different from the epidermal protease presently reported. In addition, the epidermal hydrolase is distinguishable from rat liver cathepsin H [29] and rabbit lung BANA hydrolase [17, 26]. M_r of these cysteine proteases from other organs are in the range of 26,000–28,000 and pH optima are also slightly on the acidic side. Moreover, they hydrolyzed a common protein substrate such as azocasein and histone.

Jansén and Hopsu-Havu demonstrated a pH optimum of 7.8 for one of two BANA hydrolases isolated from rat whole skin [30]. This hydrolase showed a $M_r > 300{,}000$ on Sephadex G-100 and was activated by β-mercaptoethanol and inhibited by *p*-chloromercuribenzoate. Whether or not we have purified the same enzyme is difficult to assess from available information. Recently a nonlysosomal proteolytic system has been considered to be of importance in the breakdown of endogenous proteins. A cysteine proteinase with an apparent $M_r > 400{,}000$ and most active at about pH 7.5, was purified from cytosol fraction of murine liver by Rose et al [31]. This protease was active on [^{3}H]leucine globin and detected also in kidney, brain, heart, spleen, and tumor cells. Cysteine proteinases with a high M_r were also identified in human and rat skeletal muscle [32–34] and rat cardiac muscle [35]. The epidermal aminoendopeptidase may be involved in postsynthetic changes of epidermal proteins, and investigation of native protein substrates is in progress.

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